REMARKS/ARGUMENTS

The foregoing amendments are fully supported by the specification as originally filed, and do not add new matter. Specific support for part (b) provided at least in the paragraph bridging pages 8 and 9, and at page 7, lines 13-14. Part (c) of claim 35 is supported, for example, at page 10, lines 24-28, Table 1, and page 7, lines 1-5. Newly added claim 47 is supported at least by the disclosure between page 30, line 30 and page 31, line 25. Support for new claim 48 is at least at page 5, lines 4-7, page 25, line 4 - page 29, line 33, and page 31, lines 11-18. Claim 49 is supported at least at page 31, line 26 - page 32, line 10.

Turning to the Office Action of May 26, 2004, the specification was objected to and claims 35 and 40-46 (all claims pending at the time) were rejected on various grounds.

Specification

The specification was objected to because of a typographical error at page 27, line 19, and for providing an outdated address for ATCC. The foregoing amendments correct both error, therefore, the present objection should be withdrawn.

Claim Rejections - 35 USC § 112

Enablement

Claims 35 and 40-46 were rejected because, according to the rejection, "the specification fails to provide any guidance for the successful treatment of any wounds with AL-2 polypeptides which share 85% or greater sequence homology to the amino acid sequences of SEQ ID NO: 2 and SEQ ID NO: 4." In explaining the rejection, the Examiner adds that "[i]n the absence of any guidance from the specification and in view of the variants, homologues, analogues, orthologues, fragments, muteins, isoforms which are encompassed by AL-2 polypeptides which share 85% or greater sequence homology to the amino acid sequences of SEQ ID NO: 2 and SEQ ID NO: 4, the amount of experimentation would be undue." Citing several references in support of the state of the art and alleged unpredictability in the art, the Examiner concludes that "the instant

application fails to provide adequate guidance for one skilled in the art to overcome the unpredictability and challenges of applying results from *prophetic suggestion* to the acceleration of the *neovascularization of wounds* as exemplified in the references herein." (Emphasis original.)

Without acquiescing to the rejection, or the reasoning underlying the rejection, the claims no longer recite the use of AL-2 polypeptides which share at least 85% sequence homology with the amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 4. Applicants submit that the claims currently pending are fully enabled, such that, based on the teaching of the specification, one skilled in the art could have practiced the invention at the time the invention was made, without undue experimentation.

In addition to the use of polypeptides comprising the specifically disclosed sequence of SEQ ID NO: 2 or SEQ ID NO: 4, claim 35 now recites soluble variants derived from such sequences, and mammalian homologs or conservative amino acid substitution variants that have at least 95% sequence identity with SEQ ID NO: 2 or SEQ ID NO: 4. Such limited variants can be readily made and used to promote neovascularization of a wound following the directions provided in the specification, without undue experimentation.

SEQ ID NO: 2 and SEQ ID NO: 4 represent long and short forms of a native protein originally designated AL-2 (Genentech) or EFL-6 (Regeneron), and known as a member of the ephrin ligand family, now designated ephrin-B3 (see the attached Table 1 including the current nomenclature of Eph receptors and their ligands along with their synonyms). The fact that AL-2 and ephrin-B3 are identical is evidenced by the attached printout of the ephrin-B3 amino acid sequence (Swiss-Prot Accession No. Q15968), which is identical with the sequence of the short form of AL-2.

The B-class ligands of the ephrin family are transmembrane proteins, which can be tyrosine phosphorylated upon receptor ligation. The cytoplasmic regions of ephrin ligands are highly conserved, and the ephrin ligands are promiscuous in their interaction with the Eph receptors. Thus most ligands have been shown to bind to multiple Eph receptors. For example, ephrin-B3 has been shown to bind EphA4, EphB1, EphB2 and EphB3. For more details of the Eph family of receptors and their ligands see the attached review article by Pasquale (Curr. Opin. Cell Biol., 9(5):608-5 (1997)). The role of ephrins, including the ephrin-B ligands in vascular

development is also recognized in the art. See, e.g. the attached review article by Gale and Yancopoulos, Genes & Development 13:1055-1066 (1999).

The specification of the present application provides ample teaching about methods for making amino acid variants of SEQ ID NO: 2 and SEQ ID NO: 4, and for identifying variants that are expected to work in the claimed methods. This is particularly true in view of the fact that at the priority date other, related polypeptides, such as AL-1 (now known as ephrin-A5) were known in the art, and the experience gained with those polypeptides could be used as a guidance when derivatizing AL-2. Therefore, for an ordinary artisan, who in molecular biology is expected to have a Ph.D. and several years research experience, it would not have been a particularly difficult task to prepare the variants covered by the pending claims, and used them in the claimed methods with a reasonable expectation of success.

In particular, at the priority date of the present application one of ordinary skill could have used the AL-2 nucleic acid provided by the present inventors to identify other mammalian homologues of the AL-2 sequences specifically disclosed in the present application, without undue experimentation. Indeed, murine ephrin-B3 has since been cloned found to be highly homologous with the human sequence (Swiss-Prot 035393 attached).

It is also taught in the specification, and was known at the priority date, that conservative amino acid substitutions can be made within a native sequence without compromising the desired biological activity. Possible conservative amino acid substitutions are listed in Table 1 on page 15 of the specification.

In view of the high degree of sequence identity and the known involvement of ephrin-B ligands in angiogenesis, at the priority date of this application a skilled artisan would have reasonably expected that mammalian homologues of the human sequences and conservative substitution variants having at least 95% sequence identity would be more likely than not work in the claimed methods, i.e. could be successfully used for neovascularization of wounds.

It is also taught in the specification and was well known in the art at the priority date of this application that soluble variants of naturally occurring polypeptides either retain the biological activity of the membrane-bound molecule or can be brought in a biologically active form, and thus can be used in the same treatment methods as the full-length molecule, either on

their own or in the form of fusion molecules, such as immunoadhesins, or in other clustered forms. Thus, on pages 30 and 31 of the specification, it is explained that even if the soluble form itself does not show significant biological activity (like in the AL-1), inactive soluble ligands can become biologically active when clustered, or are used in the form of ligand-IgG chimeras.

Accordingly, Applicants submit that the claims pending in this application are fully enabled, and respectfully request the reconsideration and withdrawal of the present rejection.

Written Description

Claims 35 and 40-46 were additionally rejected as allegedly failing to comply with the written description prong of 35 U.S.C. 112, first paragraph "as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention." According to the rejection, the "only adequately described species is a polypeptide comprising SEQ ID NO: 2 and 4. No active variants are disclosed." From this, the Examiner concluded that the specification does not provide adequate written description of the genus.

Applicants submit that, when assessing the indicia of patentability, including the written description requirement, the disclosure of the application as a whole must be considered through the eyes of one or ordinary skill at the effective priority date of the application. While only two specific sequences are disclosed in the present application, as discussed above, there is extensive teaching for making and using the other variants covered by the claims as currently amended.

The case law is clear that the written description requirement for a genus can be satisfied by a combination of structural and functional features. The current claims do exactly that by reciting certain structural characteristics (e.g. soluble variants, at least 95% sequence identity) coupled with the requirement of a functional feature (angiogenic activity). Accordingly, applicants have conveyed with reasonable clarity to those skilled in the art, as of the filing date sought, that they were in the possession of the invention as of that date, and the present rejection should be withdrawn.

All claims pending in this application are believed to be in *prima facie* condition for allowance, and an early action to that effect is respectfully solicited.

Please charge any additional fees, including fees for additional extension of time, or credit overpayment to Deposit Account No. **08-1641** (referencing Attorney's Docket No. **39766-0046DV1**).

Respectfully submitted,

Date: August 26, 2004

Ginger R. Dreger
Reg. No. 33,055

HELLER EHRMAN WHITE & McAULIFFE, LLP
Customer No. 25213
275 Middlefield Road
Menlo Park, California 94025
Telephone: (650) 324-7000
Facsimile: (650) 324-0638

SV 2050884 v1
8/26/04 12:51 PM (39766.0046)

NiceProt View of Swiss-Prot: Q15768

Note: most headings are clickable, even if they don't appear as links. They link to the user manual or other documents.

Entry information

Entry name	**EFB3_HUMAN**
Primary accession number	**Q15768**
Secondary accession numbers	O00680 Q8TBH7 Q92875
Entered in Swiss-Prot in	Release 35, November 1997
Sequence was last modified in	Release 35, November 1997
Annotations were last modified in	Release 45, October 2004

Name and origin of the protein

Protein name	**Ephrin-B3 [Precursor]**
Synonyms	**EPH-related receptor tyrosine kinase ligand 8** **LERK-8** **EPH-related receptor transmembrane ligand ELK-L3**
Gene name	**Name: EFNB3** Synonyms: EPLG8, LERK8
From	Homo sapiens (Human) [TaxID: 9606]
Taxonomy	Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.

References

[1] SEQUENCE FROM NUCLEIC ACID.
Cerretti D.P.;
Submitted (JUL-1996) to the EMBL/GenBank/DDBJ databases.

[2] SEQUENCE FROM NUCLEIC ACID.
TISSUE=Brain;
MEDLINE=97271551;PubMed=9126477 [NCBI, ExPASy, EBI, Israel, Japan]
Tang X.X., Pleasure D.E., Ikegaki N.;
"cDNA cloning, chromosomal localization, and expression pattern of EPLG8, a new member of the EPLG gene family encoding ligands of EPH-related protein-tyrosine kinase receptors.";
Genomics 41:17-24(1997).

[3] SEQUENCE FROM NUCLEIC ACID.
TISSUE=Brain cortex;
MEDLINE=96404527;PubMed=8808709 [NCBI, ExPASy, EBI, Israel, Japan]
Gale N.W., Flenniken A., Compton D.C., Jenkins N.A., Copeland N.G., Gilbert D.J., Davis S.,

Wilkinson D.G., Yancopoulos G.D.;
"Elk-L3, a novel transmembrane ligand for the Eph family of receptor tyrosine kinases, expressed in embryonic floor plate, roof plate and hindbrain segments.";
Oncogene 13:1343-1352(1996).

[4] SEQUENCE FROM NUCLEIC ACID.
TISSUE=Brain;
DOI=10.1073/pnas.242603899;MEDLINE=22388257;PubMed=12477932 [NCBI, ExPASy, EBI, Israel, Japan]
Strausberg R.L., Feingold E.A., Grouse L.H., Derge J.G., Klausner R.D., Collins F.S., Wagner L., Shenmen C.M., Schuler G.D., Altschul S.F., Zeeberg B., Buetow K.H., Schaefer C.F., Bhat N.K., Hopkins R.F., Jordan H., Moore T., Max S.I., Wang J., Hsieh F., Diatchenko L., Marusina K., Farmer A.A., Rubin G.M., Hong L., Stapleton M., Soares M.B., Bonaldo M.F., Casavant T.L., Scheetz T.E., Brownstein M.J., Usdin T.B., Toshiyuki S., Carninci P., Prange C., Raha S.S., Loquellano N.A., Peters G.J., Abramson R.D., Mullahy S.J., Bosak S.A., McEwan P.J., McKernan K.J., Malek J.A., Gunaratne P.H., Richards S., Worley K.C., Hale S., Garcia A.M., Gay L.J., Hulyk S.W., Villalon D.K., Muzny D.M., Sodergren E.J., Lu X., Gibbs R.A., Fahey J., Helton E., Ketteman M., Madan A., Rodrigues S., Sanchez A., Whiting M., Madan A., Young A.C., Shevchenko Y., Bouffard G.G., Blakesley R.W., Touchman J.W., Green E.D., Dickson M.C., Rodriguez A.C., Grimwood J., Schmutz J., Myers R.M., Butterfield Y.S.N., Krzywinski M.I., Skalska U., Smailus D.E., Schnerch A., Schein J.E., Jones S.J.M., Marra M.A.;
"Generation and initial analysis of more than 15,000 full-length human and mouse cDNA sequences.";
Proc. Natl. Acad. Sci. U.S.A. 99:16899-16903(2002).

[5] SEQUENCE OF 28-42.
Zhang Z., Henzel W.;
"Signal peptide prediction based on analysis of experimentally verified cleavage sites.";
Submitted (JUN-2004) to Swiss-Prot.

[6] INTERACTIONS WITH GRIP1 AND GRIP2.
TISSUE=Fetal brain;
MEDLINE=99211388;PubMed=10197531 [NCBI, ExPASy, EBI, Israel, Japan]
Brueckner K., Labrador J.P., Scheiffele P., Herb A., Seeburg P.H., Klein R.;
"Ephrin-B ligands recruit GRIP family PDZ adaptor proteins into raft membrane microdomains.";
Neuron 22:511-524(1999).

Comments

- ***FUNCTION***: May play a pivotal role in forebrain function. Binds to, and induce the collapse of, commissural axons/growth cones in vitro. May play a role in constraining the orientation of longitudinally projecting axons *(By similarity)*.
- ***SUBUNIT***: Interacts with GRIP1 and GRIP2.
- ***SUBCELLULAR LOCATION***: Type I membrane protein.
- ***TISSUE SPECIFICITY***: Highly expressed in brain; expressed in embryonic floor plate, roof plate and hindbrain segments.
- ***SIMILARITY***: Belongs to the ephrin family.

Copyright

Cross-references

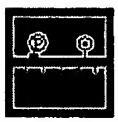
U57001; AAB05170.1; -. [EMBL / GenBank / DDBJ] [CoDingSequence]
U66406; AAC51203.1; -. [EMBL / GenBank / DDBJ] [CoDingSequence]

EMBL	U62775; AAC50707.1; -. [EMBL / GenBank / DDBJ] [CoDingSequence] BC022499; AAH22499.1; -.[EMBL / GenBank / DDBJ] [CoDingSequence] BC042944; AAH42944.1; -.[EMBL / GenBank / DDBJ] [CoDingSequence]
HSSP	P52800; 1IKO. [HSSP ENTRY / PDB]
Genew	HGNC:3228; EFNB3.
CleanEx	HGNC:3228; EFNB3.
GeneCards	EFNB3.
GeneLynx	EFNB3; Homo sapiens.
GenAtlas	EFNB3.
MIM	602297 [NCBI / EBI].
GO	GO:0005887;Cellular component: integral to plasma membrane *(traceable author statement)*. GO:0005005;Molecular function: transmembrane-ephrin receptor activity *(traceable author statement)*. GO:0007267;Biological process: cell-cell signaling *(traceable author statement)*. GO:0007399;Biological process: neurogenesis *(traceable author statement)*.
SOURCE	EFNB3; Homo sapiens.
Ensembl	Q15768; Homo sapiens. [Entry / Contig view]
InterPro	IPR008972; Cupredoxin. IPR001799; Ephrin. Graphical view of domain structure.
Pfam	PF00812; Ephrin; 1. Pfam graphical view of domain structure.
PRINTS	PR01347; EPHRIN.
ProDom	PD002533; Ephrin; 1. [Domain structure / List of seq. sharing at least 1 domain]
PROSITE	PS01299; EPHRIN; 1.
HOVERGEN	[Family / Alignment / Tree]
BLOCKS	Q15768.
ProtoNet	Q15768.
ProtoMap	Q15768.
PRESAGE	Q15768.
DIP	Q15768.
ModBase	Q15768.
SMR	Q15768; EDFF2A23C2FDE79F.
SWISS-2DPAGE	Get region on 2D PAGE.
UniRef	View cluster of proteins with at least 50% / 90% identity.

Keywords

Developmental protein; **Direct protein sequencing**; **Glycoprotein**; **Neurogenesis**; **Polymorphism**; **Signal**; **Transmembrane**.

Features

 Feature table viewer

 Feature aligner

Key	From	To	Length	Description	FTId
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DOMAIN	28	226	199	Extracellular (*Potential*).	
TRANSMEM	227	247	21	*Potential*.	
DOMAIN	248	340	93	Cytoplasmic (*Potential*).	
DOMAIN	338	340	3	PDZ RECOGNITION MOTIF (*POTENTIAL*).	
DISULFID	62	104		*By similarity*.	
DISULFID	92	156		*By similarity*.	
CARBOHYD	210	210		N-linked (GlcNAc...) (*Potential*).	
VARIANT	166	166	*	R -> Q.	VAR_002356

Sequence information

Length: **340 AA** [This is the length of the unprocessed precursor]

Molecular weight: **35834 Da** [This is the MW of the unprocessed precursor]

CRC64: **EDFF2A23C2FDE79F** [This is a checksum on the sequence]

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        70         80         90        100        110        120
         |          |          |          |          |          |
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       130        140        150        160        170        180
         |          |          |          |          |          |
SPNLWGHEFR SHHDYYIIAT SDGTREGLES LQGGVCLTRG MKVLLRVGQS PRGGAVPRKP

       190        200        210        220        230        240
         |          |          |          |          |          |
VSEMPMERDR GAAHSLEPGK ENLPGDPTSN ATSRGAEGPL PPPSMPAVAG AAGGLALLLL

       250        260        270        280        290        300
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GVAGAGGAMC WRRRRAKPSE SRHPGPGSFG RGGSLGLGGG GGMGPREAEP GELGIALRGG

       310        320        330        340
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GAADPPFCPH YEKVSGDYGH PVYIVQDGPP QSPPNIYYKV
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Sequence analysis tools: ProtParam, ProtScale, Compute pI/Mw, PeptideMass, PeptideCutter, Dotlet (Java)

ScanProsite, MotifScan

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Entry information

Entry name	**EFB3_MOUSE**
Primary accession number	**O35393**
Secondary accession numbers	None
Entered in Swiss-Prot in	Release 38, July 1999
Sequence was last modified in	Release 38, July 1999
Annotations were last modified in	Release 44, July 2004

Name and origin of the protein

Protein name	**Ephrin-B3 [Precursor]**
Synonyms	None
Gene name	**Name: Efnb3**
From	Mus musculus (Mouse) [TaxID: 10090]
Taxonomy	Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Rodentia; Sciurognathi; Muridae; Murinae; Mus.

References

[1] SEQUENCE FROM NUCLEIC ACID.
TISSUE=Brain;
MEDLINE=98143367;PubMed=9484836 [NCBI, ExPASy, EBI, Israel, Japan]
Bergemann A.D., Zhang L., Chiang M.-K., Brambilla R., Klein R., Flanagan J.G.;
"Ephrin-B3, a ligand for the receptor EphB3, expressed at the midline of the developing neural tube.";
Oncogene 16:471-480(1998).

[2] SEQUENCE FROM NUCLEIC ACID.
STRAIN=C57BL/6;
TISSUE=Brain;
DOI=10.1073/pnas.242603899;MEDLINE=22388257;PubMed=12477932 [NCBI, ExPASy, EBI, Israel, Japan]
Strausberg R.L., Feingold E.A., Grouse L.H., Derge J.G., Klausner R.D., Collins F.S., Wagner L., Shenmen C.M., Schuler G.D., Altschul S.F., Zeeberg B., Buetow K.H., Schaefer C.F., Bhat N.K., Hopkins R.F., Jordan H., Moore T., Max S.I., Wang J., Hsieh F., Diatchenko L., Marusina K., Farmer A.A., Rubin G.M., Hong L., Stapleton M., Soares M.B., Bonaldo M.F., Casavant T.L.,

Scheetz T.E., Brownstein M.J., Usdin T.B., Toshiyuki S., Carninci P., Prange C., Raha S.S., Loquellano N.A., Peters G.J., Abramson R.D., Mullahy S.J., Bosak S.A., McEwan P.J., McKernan K.J., Malek J.A., Gunaratne P.H., Richards S., Worley K.C., Hale S., Garcia A.M., Gay L.J., Hulyk S.W., Villalon D.K., Muzny D.M., Sodergren E.J., Lu X., Gibbs R.A., Fahey J., Helton E., Ketteman M., Madan A., Rodrigues S., Sanchez A., Whiting M., Madan A., Young A.C., Shevchenko Y., Bouffard G.G., Blakesley R.W., Touchman J.W., Green E.D., Dickson M.C., Rodriguez A.C., Grimwood J., Schmutz J., Myers R.M., Butterfield Y.S.N., Krzywinski M.I., Skalska U., Smailus D.E., Schnerch A., Schein J.E., Jones S.J.M., Marra M.A.;
"Generation and initial analysis of more than 15,000 full-length human and mouse cDNA sequences.";
Proc. Natl. Acad. Sci. U.S.A. 99:16899-16903(2002).

[3] FUNCTION.
MEDLINE=20171264;PubMed=10704386 [NCBI, ExPASy, EBI, Israel, Japan]
Imondi R., Wideman C., Kaprielian Z.;
"Complementary expression of transmembrane ephrins and their receptors in the mouse spinal cord: a possible role in constraining the orientation of longitudinally projecting axons.";
Development 127:1397-1410(2000).

Comments

- ***FUNCTION***: May play a pivotal role in forebrain function. Binds to, and induce the collapse of, commissural axons/growth cones in vitro. May play a role in constraining the orientation of longitudinally projecting axons.
- ***SUBUNIT***: Interacts with GRIP1 and GRIP2 *(By similarity)*.
- ***SUBCELLULAR LOCATION***: Type I membrane protein.
- ***TISSUE SPECIFICITY***: Expressed on lateral floor plate cells, specifically on commissural axon segments that have passed through the floor plate. Expressed in cells of the retinal ganglion cell layer during retinal axon guidance to the optic disk.
- ***DEVELOPMENTAL STAGE***: Expressed in the floor plate throughout the period of commissural axon pathfinding.
- ***SIMILARITY***: Belongs to the ephrin family.

Copyright

Cross-references

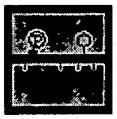
EMBL	AF025288; AAC53537.1; -.[EMBL / GenBank / DDBJ] [CoDingSequence] BC052001; AAH52001.1; -.[EMBL / GenBank / DDBJ] [CoDingSequence] BC058617; AAH58617.1; -.[EMBL / GenBank / DDBJ] [CoDingSequence]
HSSP	P52800; 1IKO. [HSSP ENTRY / PDB]
MGD	MGI:109196; Efnb3.
GeneLynx	Efnb3; Mus musculus.
GO	GO:0007628;Biological process: adult walking behavior *(inferred from mutant phenotype)*. GO:0007411;Biological process: axon guidance *(inferred from mutant phenotype)*.
SOURCE	Efnb3; Mus musculus.
Ensembl	O35393; Mus musculus. [Entry / Contig view]
InterPro	IPR008972; Cupredoxin. IPR001799; Ephrin. Graphical view of domain structure.

Pfam	PF00812; Ephrin; 1. Pfam graphical view of domain structure.
PRINTS	PR01347; EPHRIN.
ProDom	PD002533; Ephrin; 1. [Domain structure / List of seq. sharing at least 1 domain]
PROSITE	PS01299; EPHRIN; 1.
HOVERGEN	[Family / Alignment / Tree]
BLOCKS	O35393.
ProtoNet	O35393.
ProtoMap	O35393.
PRESAGE	O35393.
DIP	O35393.
ModBase	O35393.
SMR	O35393; 52F3D58FD209A6B8.
SWISS-2DPAGE	Get region on 2D PAGE.
UniRef	View cluster of proteins with at least 50% / 90% identity.

Keywords

Developmental protein; **Glycoprotein**; **Neurogenesis**; **Signal**; **Transmembrane**.

Features

 Feature table viewer

 Feature aligner

Key	From	To	Length	Description
SIGNAL	1	27	27	*Potential.*
CHAIN	28	340	313	Ephrin-B3.
DOMAIN	28	227	200	Extracellular (*Potential*).
TRANSMEM	228	248	21	*Potential.*
DOMAIN	249	340	92	Cytoplasmic (*Potential*).
DOMAIN	338	340	3	PDZ RECOGNITION MOTIF (*POTENTIAL*).
DISULFID	62	104		*By similarity.*
DISULFID	92	156		*By similarity.*
CARBOHYD	210	210		N-linked (GlcNAc...) (*Potential*).

Sequence information

Length: **340 AA** [This is the length of the unprocessed precursor]

Molecular weight: **35884 Da** [This is the MW of the unprocessed precursor]

CRC64: **52F3D58FD209A6B8** [This is a checksum on the sequence]

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MGAPHFGPGG VQVGALLLLG FAGLVSGLSL EPVYWNSANK RFQAEGGYVL YPQIGDRLDL

        70          80          90         100         110         120
         |           |           |           |           |           |
LCPRARPPGP HSSPSYEFYK LYLVEGAQGR RCEAPPAPNL LLTCDRPDLD LRFTIKFQEY

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SPNLWGHEFR SHHDYYIIAT SDGTREGLES LQGGVCLTRG MKVLLRVGQS PRGGAVPRKP

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VSEMPMERDR GAAHSAEPGR DTIPGDPSSN ATSRGAEGPL PPPSMPAVAG AAGGMALLLL

       250        260        270        280        290        300
        |          |          |          |          |          |
GVAGAGGAMC WRRRRAKPSE SRHPGPGSFG RGGSLGLGGG GGMGPREAEP GELGIALRGG

       310        320        330        340
        |          |          |          |
GTADPPFCPH YEKVSGDYGH PVYIVQDGPP QSPPNIYYKV
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ScanProsite, MotifScan

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60d

The Eph family of receptors

Elena B Pasquale

Eph receptor tyrosine kinases have recently been identified as instructive molecules that guide the topographic movement of cells and growth cones. The activation of Eph receptors by their ligands, which are membrane-anchored molecules, involves a cell–cell recognition event that often causes cell repulsion. Therefore, Eph receptors mediate signals that can override cell adhesion. Transmembrane ligands for Eph receptors also exhibit properties of signal transducing molecules, suggesting that bidirectional signaling occurs when receptor-expressing cells contact ligand-expressing cells.

Addresses
The Burnham Institute, 10901 N Torrey Pines Road, La Jolla, CA 92037, USA; e-mail: elenap@ljcrf.edu

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Abbreviations

GAP	GTPase-activating protein
GPI	glycosylphosphatidylinositol
PI 3-kinase	phosphatidylinositol 3-kinase
SH2	Src homology 2
SLAP	Src-like adaptor protein

Introduction

Directional movement of cells and cell processes requires molecules with permissive and instructive functions [1]. Recently identified families of instructive molecules include the netrins, semaphorins, and Eph family receptors and ligands [1]. In this review, I summarize new developments in the study of the role of Eph receptors in contact-mediated cell guidance.

The Eph family comprises fourteen structurally related transmembrane receptor tyrosine kinases (Table 1). The extracellular region of Eph receptors is composed of a series of modules, namely, a putative immunoglobulin (Ig) domain at the amino terminus, followed by a cysteine-rich region and two fibronectin type III repeats near the single membrane-spanning segment. The cytoplasmic region contains a highly conserved tyrosine kinase domain flanked by a juxtamembrane region and a carboxy-terminal tail, which are less conserved [2]. Many receptor variant forms also exist that do not conform to the prototypical domain structure, as they contain deletions, truncations, substitutions, or insertions (see [3] and references therein). One Eph receptor, Mep, lacks kinase activity [4].

Because so many Eph receptor genes have been identified in different species within a brief period of time, each gene has been assigned multiple names (Table 1). According to a unified nomenclature, which has been published elsewhere [5], the Eph receptors are divided into two groups on the basis of sequence homologies. Receptors of the EphA group preferentially interact with glycosylphosphatidylinositol (GPI)-linked ligands (of the ephrin-A subclass, which comprises five ligands), while receptors of the EphB group preferentially interact with transmembrane ligands (of the ephrin-B subclass, which comprises three ligands) (Table 1). In either case, binding of a ligand results in Eph receptor autophosphorylation on tyrosine residues and activation of the kinase activity of the Eph receptor [6–8].

Spatially restricted Eph receptor activation

As, *in vitro*, each Eph receptor binds multiple ligands and each ligand binds multiple receptors, determination of the expression patterns of the Eph receptors and ligands is crucial to identifying which interactions occur *in vivo*. In addition, it provides clues to how the Eph receptors and their ligands function.

There are numerous examples of reciprocal expression of Eph receptors and their ligands (summarized in Table 2 and shown schematically in Figure 1a,b). Reciprocal distributions allow for only limited receptor activation, restricted to sites where ligand and receptor come into contact in significant numbers. Recent evidence suggests that Eph receptors guide the movement of growth cones and cells in which they are expressed in order to avoid regions of excessive ligand concentration, thereby influencing axon pathfinding and the segregation of subpopulations of cells. As discussed below, repulsive interactions between receptor- and ligand-expressing cells are presumably responsible for these activities.

An example of Eph receptor mediated axon guidance is the specification of retinotectal topography. Even before the Eph receptors were found to be involved in this process, it was established that the precise targeting of retinal ganglion cell axons in the optic tectum relies upon repulsive activities mediated by molecules expressed in a graded manner on the ingrowing retinal axons and in the tectum [9,10]. Consistent with this model, retinal ganglion cells from different portions of the retina express different levels of the Eph receptor Cek4 (Table 2) [11]. Temporal retinal axons, which have high levels of Cek4 expression, grow to the anterior tectum, where expression of the two GPI-linked ligands, ELF1 and RAGS (see Table 1), is low [11,12]. These two ligands are distributed in superimposed increasing anterior to posterior gradients in the tectum, with the higher affinity ligand for Cek4, RAGS, restricted to more posterior regions than the lower affinity ligand, ELF1 [13]. *In vitro* experiments have confirmed that both ELF1 and RAGS behave as repulsive guidance molecules.

Table 1

The Eph receptors and their ligand specificities.

Receptors					Ligands*
Unified nomenclature	Human	Mouse	Rat	Chicken/Quail	
EphA receptors					
EphA1	Eph	Esk	–	–	Ephrin-A1
EphA2	Eck	mEck/Myk2/Sek2	–	–	Ephrin-A3, -A1, -A5, -A4
EphA3	Hek	Mek4	Tyro4	Cek4	Ephrin-A5, -A2, -A3, -A1
EphA4	Hek8	Sek1	Tyro1	Cek8	Ephrin-A5, -A1, -A3, -A2, -B2, -B3
EphA5	Hek7	Bsk	Ehk1/Rek7	Cek7	Ephrin-A5, -A1, -A2, -A3, -A4
EphA6	–	mEhk2	Ehk2	–	Ephrin-A2, -A1, -A3, -A4, -A5
EphA7	Hek11	Mdk1/Ebk	Ehk3	–	Ephrin-A2, -A3, -A1
EphA8	Eek	mEek	Eek	–	Ephrin-A5, -A3, -A2
EphB receptors					
EphB1	Net	–	Elk	Cek6	Ephrin-B2, -B1, -A3
EphB2	Erk/Hek5/Drt	Nuk/Sek3	Tyro5	Cek5, Qek5	Ephrin-B1, -B2, -B3
EphB3	Hek2	Sek4/Mdk5	Tyro6	Cek10, Qek10	Ephrin-B1, -B2, -B3
EphB4	Htk	Myk1/Mdk2	Tyro11	–	Ephrin-B2, -B1
EphB5	–	–	–	Cek9	?
EphB6	Hep	Mep	–	–	?

*Ligands are listed in approximate order of decreasing affinity for receptor according to a recently published unified nomenclature [5]. Other names for these ligands are as follows: ephrin-A1, B61; ephrin-A2, ELF1; ephrin-A3, Ehk1-L, Lerk3; ephrin-A4, Lerk4; ephrin-A5, AL1, RAGS; ephrin-B1, Lerk2, Elk-L, Cek5-L; ephrin-B2, Htk-L, ELF2, Lerk5; ephrin-B3, NLerk2, Elk-L3. Please note that not all possible ligand–receptor interactions have been tested. Long dashes indicate that homologs of some receptors in other species have not yet been found. Question marks indicate that the ligands for Cek9 and Hep/Mep are unknown.

The growth cones of cultured temporal retinal neurons collapse when exposed to soluble forms of these ligands, and in 'stripe assays' temporal retinal axons, grown on stripes containing RAGS or ELF1 alternating with stripes without ligand, avoid extending on the stripes coated with ligand [12,13,14••]. The *in vivo* repulsive activity of ELF1 was demonstrated when the distribution of ELF1 in the chicken tectum was modified by retroviral expression: temporal axons followed aberrant trajectories that avoided ectopic areas of abnormally high ELF1 expression in the anterior tectum [14••].

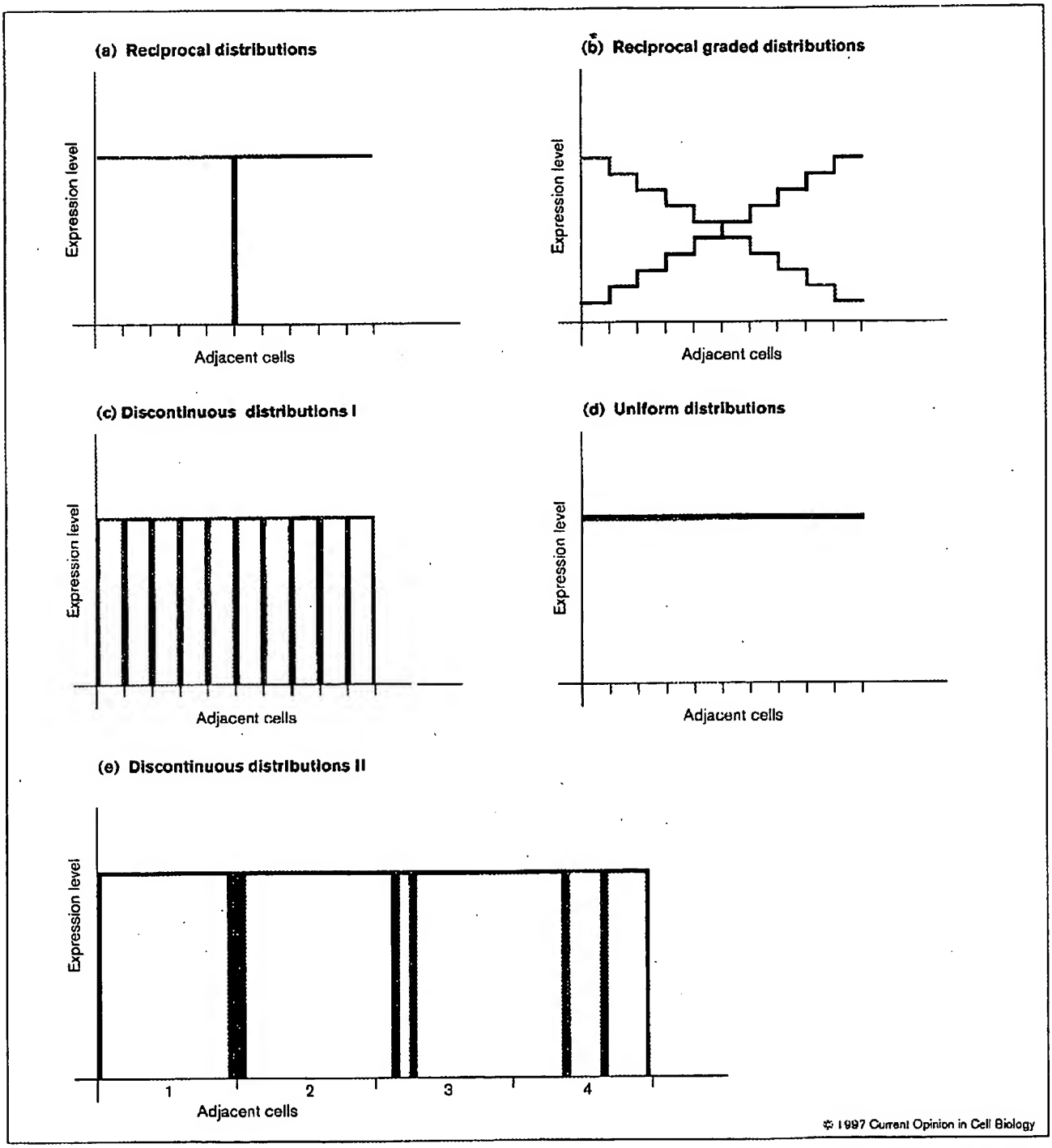
Other examples where the reciprocal expression patterns of Eph receptors and their ligands suggest that Eph receptors and ligands guide axons by repulsive mechanisms are summarized in the top half of Table 2. Interestingly, the confinement of spinal axon growth to rostral somite halves had been attributed to repulsive activities located in caudal somite halves [15]. As it turns out, the transmembrane Eph receptor ligands Htk-L and Lerk2 (see Table 1) are restricted to caudal somite halves [16•,17••], whereas several Eph receptors, including Nuk/Cek5 and Cek8, are found on axons of spinal neurons [7,17••,18]. Repulsive activities of these transmembrane ligands (Htk-L and Lerk2) toward axons extending from neural tube explants has been demonstrated *in vitro* in both growth cone collapse assays and stripe assays [17••].

Examples of where Eph receptors and their ligands are expressed in adjacent populations of cells, suggesting that they restrict cell movement across boundaries of ligand and receptor expression, are summarized in the bottom half of Table 2. Experimental evidence supports a role for Eph receptors in restricting and guiding cell migration. Exposure of chicken embryo trunk explants to a soluble dimeric form of the ligand Elk-L (see Table 1) perturbed the routing of trunk neural crest cells, which express a receptor, Qek10/Cek10, for Elk-L [19•]. Although the motility of neural crest cells was not appreciably affected, their movement no longer exhibited a persistent directional component, nor was it restricted to the normal route across rostral somite halves. Similarly, in *Xenopus* embryos the migration of third branchial arch neural crest cells, which express the receptors Sek1 and Elk, was perturbed by the ectopic uniform expression of a ligand for these receptors, Htk-L, which is normally restricted to the adjacent second arch neural crest [20•]. Ectopic expression in *Xenopus* embryos of dominant-negative forms of the receptors Sek1 and Elk, which lacked the kinase domain and were thus unable to transduce signals, also interfered with the directional migration of third arch neural crest cells. Furthermore, the boundaries of rhombomeres 3 and 5, which express Sek1, became blurred in the presence of ectopic dominant-negative Sek1 [21].

Persistent Eph receptor activation

Overlapping distributions of Eph receptors and their ligands have also been observed; this may cause persistent receptor activation. For example, in the 11-day chicken embryonic retina the receptor Cek5 and its ligand, Cek5-L, have overlapping expression patterns; binding of Cek5-L to Cek5 results in enhanced Cek5 phosphorylation on tyrosine [22,23]. During the first half of chicken embryonic development, Cek9 is highly phosphorylated on tyrosine, possibly reflecting extensive interactions with its as yet unidentified ligand [24]. In the developing

Figure 1



Schematic representation of the possible relative distributions of Eph receptors and their ligands in a series of adjacent cells. Each subdivision on the X axis indicates a cell; the Y axis indicates the level of ligand or receptor expression, in arbitrary units. Ligand expression levels are indicated in gray and receptor expression levels in black. **(a,b)** Illustrate reciprocal and reciprocal graded patterns of expression, respectively. **(c–e)** Illustrate hypothetical distributions in tissues in which receptor and ligand have overlapping expression patterns. In (c) each cell expresses either receptor or ligand, and receptor-bearing cells and ligand-bearing cells are interspersed. In (d) each cell expresses both receptor and ligand, and receptor and ligand have uniform distribution over the surface of the cell. In (e) each cell (depicted at higher magnification and indicated by a number) expresses both receptor and ligand, but receptor and ligand do not co-localize. Rather, they are confined to separate cell surface domains: for example, the ligand is in the region of contact between cell 1 and cell 2 and in subdomains of cell 3 and cell 4 that could represent a presynaptic or postsynaptic region. In contrast, the receptor would be on the extrasynaptic portions of the cell surface. High resolution localization experiments will be required to determine which of the distributions shown in (c–e) actually occurs *in vivo*.

Table 2

Examples of reciprocal expression patterns of Eph family receptors and ligands.

Receptor(s)	Receptor distribution	Ligand(s)	Ligand distribution	Proposed function	References
Cek4	Increasing nasal to temporal gradient in the developing retina	ELF1, RAGS	Increasing anterior to posterior gradients in the developing optic tectum	Targeting of retinotectal projection	[11–13,14••]
Bek	Increasing lateral to medial gradient in the developing and adult hippocampus	ELF1, RAGS, Lerk3	Increasing dorsomedial to ventrolateral gradient (of the three ligands considered in combination) in the developing and adult lateral septum	Targeting and remodeling of hippocamposeptal projection	[45•]
Bsk	Increasing ventral to dorsal gradient in the subiculum	AL1	Increasing caudal to rostral gradient in the hypothalamus	Targeting of projections from subiculum to hypothalamus	[45•]
Bsk	Subpopulation of mitral and tufted cells in the embryonic and adult olfactory bulb	Lerk3	Subpopulation of olfactory sensory neurons in the nasal epithelium	Specification of synapses between olfactory neurons and mitral or tufted cells	[45•]
?	Caudally derived (lumbar) sensory and motor axons?	AL1	Higher expression in rostral (head and neck) embryonic muscles than in caudal (trunk and limb) muscles	Regulating position-dependent connectivity along the rostrocaudal axis between spinal cord axons and target muscles	[26]
Cek8	Lumbar and brachial embryonic motor neurons	?	?	Guiding lumbar and brachial embryonic motor neurons to their target muscles	[46]
Cek4/Mek4/Tyro4	Cervical and thoracic motor neurons of the medial motor column. These neurons innervate receptor-positive axial musculature	ELF1?	Limb buds	ELF1 may repel receptor-positive myoblasts and axons away from the limb bud and direct them jointly into the body wall	[47]
Nuk	Brain regions immediately ventral to the anterior commissure	Lerk2	Axons of the posterior portion of the anterior commissure	Guiding the axons of the posterior portion of the anterior commissure	[42••]
Nuk/Cek5, Cek8	Spinal nerves	Lerk2, Htk-L	Caudal somite halves	Restricting the growth of spinal nerves to the rostral somite halves	[7,16•,17••,18]
Nuk, Cek10/Qek10	Trunk neural crest cells and rostral somite halves	Elk-L/Lerk2, Htk-L	Caudal somite halves	Restricting the migration of trunk neural crest cells to the rostral somite halves	[17••,19•,25]
Sek1, Elk	Third branchial arch neural crest cells and mesoderm along their presumptive migratory pathways	Htk-L	Second branchial arch neural crest cells and mesoderm along their presumptive migratory pathways	Guiding the migration of third branchial arch neural crest cells	[20•]
Sek1, Nuk	Hindbrain rhombomeres 3 and 5	Transmembrane ligands	Hindbrain rhombomeres 2, 4, and 6	Defining rhombomere boundaries	[18,21,25,48–50]
EphA receptors	Mouse E10.5 limb: distal tip (prospective autopod) and body-proximal areas (prospective stylopod)•	GPI-linked ligands	Mouse E10.5 limb: central portion (prospective zeugopod)•	Limb patterning: specification of domains with different fates	[16•]
EphA receptors	Mouse E13 limb: cartilagenous digits•	GPI-linked ligands	Mouse E13 limb: interdigital zone•	Limb patterning: segregation of condensing cartilage from surrounding mesenchyme	[16•,25,51,52]
Cek8	E8 chicken ventral retina•	Cek5-L	E8 chicken dorsal retina•	Segregating dorsal and ventral retinal cells, organizing the retinotectal pathway	[23,53]
Cek4	Embryonic temporal retina	RAGS	Embryonic nasal retina•	Segregating nasal and temporal retinal cells, organizing the retinotectal pathway	[25,54] (a)
Htk	Erythroid progenitor cells	Htk-L	Bone marrow stromal cells	Regulating erythropoiesis by providing positional information in the bone marrow microenvironment	[44]

(a) RJ Connor, P Menzel, EB Pasquale, unpublished data. •E refers to embryonic day of development. Question marks indicate that receptor, ligand or ligand distribution is not yet known. Examples where the reciprocal expression patterns of Eph receptors and their ligands suggest that Eph receptors and ligands guide axons by repulsive mechanisms are shown in the top half of the table. Examples where the Eph receptors and their ligands are expressed in adjacent populations of cells, suggesting that they restrict cell movement across boundaries of ligand and receptor expression, are shown in the bottom half of the table.

mouse embryo, expression of the receptor Sek1 in the dorsal portion of the somites overlaps with that of the ligands AL1 and Lerk4, which are localized throughout the somites [25]. It is not known, however, whether in regions of overlapping expression ligand and receptor are found on distinct but mixed subpopulations of cells (Figure 1c), or on the same cells either with uniform distribution over the surface of the cell (Figure 1d) or confined to complementary subcellular domains (Figure 1e).

Uniform ligand concentrations should presumably provide signals other than directional cues and their effects may be more subtle. Uniformly presented ligands did not prevent the growth of neurites from retinal or neural tube explants [14••,17••] nor did they prevent *in vitro* or *in vivo* motility of trunk neural crest cells [17••,19•]. However, the GPI-linked ligands AL1 and ELF1, expressed on a continuous monolayer of cells, appreciably inhibited outgrowth of neurites from neurons expressing the receptors [26,27]. Effects on axon fasciculation and cell–cell adhesion have also been demonstrated. Soluble AL1 ligand promoted axon fasciculation in cultures of cortical neurons, which express the Eph receptor Rek7 [28]. Ectopic expression of an activated form of the receptor Pag (*Xenopus* Sek1) in *Xenopus* embryos caused decreased cell–cell adhesion, possibly by affecting cadherin function [29]. Effects of Eph receptors such as Cek5 on neuronal recognition events or fasciculation could result from the tyrosine phosphorylation of the neural cell adhesion molecule L1 [30]. However, the functional consequences of L1 phosphorylation by Cek5 remain to be determined.

Activation of Eph receptors may also be relevant in angiogenesis. When activated by their ligands, the re-

ceptors Eck and Elk mediate the *in vitro* assembly of human umbilical vein endothelial cells and human renal microvascular endothelial cells, respectively, into capillary-like structures [31,32]. Furthermore, a soluble dimeric form of the Eck ligand, B61, has been shown to act as an angiogenic factor *in vivo* [32].

Receptor signaling

Cell–cell recognition mediated by Eph receptors results in signals that override adhesive mechanisms mediated by cell adhesion molecules, integrins, and possibly the receptor–ligand interaction itself [33]. Furthermore, growth cone collapse induced by ligands for Eph receptors such as AL1 is accompanied by disruption of the actin cytoskeleton in the growth cone [34]. In contrast, proliferative signals generated by Eph receptors are at best modest [35]. Like other receptor tyrosine kinases, the Eph receptors autophosphorylate in response to ligand binding [2]. Only membrane-anchored ligands, however, efficiently activate their receptors [6]. Monomeric soluble ligands that are released into the culture medium generally do not cause receptor signaling, even though they retain high binding affinity [6].

A number of signaling molecules that bind by means of their SH2 (Src homology 2) domains to tyrosine-phosphorylated sequences of Eph receptors have been identified (Table 3). Their associations with Eph receptors have been demonstrated by using the two-hybrid system and/or biochemical assays. Grb2, Grb10, and Src-like adaptor protein (SLAP) are adaptor molecules that presumably link Eph receptors to downstream signaling pathways, such as, in the case of Grb2, the Ras pathway [2]. Phosphatidylinositol 3-kinase (PI 3-kinase), Src family kinases, and p120Ras GTPase-activating protein (GAP) have intrinsic catalytic activities. PI 3-kinase activity is increased following activation of the receptor Eck [36], but it is not known whether the catalytic activities of Src and p120RasGAP are affected by activation of the Eph receptors to which they bind.

It appears that the Eph receptors engage downstream molecules that were previously known as components in signaling pathways of other families of receptor tyrosine kinases, such as growth factor receptors [2]. The only novel protein identified so far by virtue of its interaction with an Eph receptor is SLAP [37]. Src family kinases, PI 3-kinase, and p120RasGAP have been previously implicated in neurite outgrowth, cell migration, and cytoskeletal organization (Table 3). In particular, given their co-localization with Eph receptors in growth cones and axons [38], Src family kinases are promising candidates as mediators of signals involved in axon guidance.

Ligand signaling?

Three ligands for Eph receptors, Lerk2, Htk-L, and Elk-L3/NLerk2, contain a transmembrane segment and a highly conserved cytoplasmic region of approximately 80 amino acids. Evidence has suggested that the cytoplasmic domain of the ligand Lerk2, although not required for receptor activation, has signaling activities, as it inhibits signaling pathways activated by tyrosine kinases [39,40•]. Tyrosine phosphorylation of the ligands Lerk2 and Htk-L has indeed been observed *in vivo* in the mouse embryo, demonstrating the physiological relevance of such phosphorylation, and has also been observed in actively growing cultured fibroblasts and v-Src-transformed cells [40•,41••]. Tyrosine phosphorylation of transmembrane ligands for Eph receptors is also rapidly upregulated by activation of growth factor receptors and, notably, upon interaction with EphB receptors. Ligand phosphorylation is presumably mediated by an as yet unknown ligand-associated tyrosine kinase and may promote the formation of complexes of ligands with SH2 and phosphotyrosine-binding domains of signaling molecules. Hence, contact between cells expressing ligand and cells expressing receptor probably results in activation of bidirectional signaling pathways.

Genetic analyses of Eph receptor functions

Gene knockout experiments substantiated the importance of Eph receptors in axon guidance and fasciculation. Disruption of the Nuk receptor gene affected axons of the anterior commissure, which did not follow their normal route to the contralateral side of the brain and incorrectly grew toward the ventral forebrain [42••]. Disruption of the closely related Sek4 receptor gene affected the axons of the corpus callosum, which did not follow their normal

Table 3

Signaling molecules that bind to Eph receptors through their SH2 domains.

Signaling molecule	Eph receptor	Binding site in Eph receptor	Possible role of signaling molecule in	Reference
p85 subunit of PI 3-kinase	Eck	n.d.	Cell migration, cytoskeletal organization	[36]
SLAP	Eck	n.d.	n.d.	[37]
Grb10	Elk	Tyr929	Neuronal cell migration	[55]
Grb2	Elk	In kinase domain	Neuronal differentiation, linkage to Ras pathway	[55]
Fyn	Sek1	Tyr602	Cytoskeletal organization	[56]
Src	Cek5, Cek8	Tyr611 of Cek5	Cytoskeletal organization	(a)
p120RasGAP	Nuk	Tyr604, Tyr610	Cytoskeletal organization, linkage to Ras pathway	[57]

(a) AH Zisch, MS Kalo, EB Pasquale, unpublished data. n.d., not determined.

trajectories to the contralateral side of the brain [43••]. In the absence of both Nuk and Sek4, pathfinding abnormalities were more severe, and other brain projections were also abnormal, indicating that the receptors Nuk and Sek4 have partially redundant roles [42••,43••]. Interestingly, in at least one axon tract—the habenular–interpeduncular tract, in which transmembrane ligands are co-expressed with Nuk and Sek4 receptors—axon fasciculation rather than pathfinding was affected.

Genetic evidence also supports the physiological relevance of signaling through the cytoplasmic domain of ligands [42••]. The axons of the brain anterior commissure, which are abnormal in Nuk knockout mice, were surprisingly found to express not Nuk but the Nuk ligand Lerk2. Interestingly, areas of Nuk expression are adjacent to the pathway followed by these axons (Table 2), suggesting that Nuk provides guidance cues that are transduced through the cytoplasmic domain of Lerk2. Consistently, Nuk mutant receptors lacking the kinase domain are able to properly guide anterior commissure axons [42••].

Conclusions and perspectives

What we have learnt so far about the distributions and activities of Eph family receptors and their ligands predicts that their roles are not restricted to developmental processes. Their expression in adult neural structures, such as the hippocampus and olfactory bulb, suggests a role in synaptic remodeling. The repulsive activities of ligands toward axons expressing Eph receptors predicts that these ligands may contribute to the failure of the adult central nervous system to regenerate. Finally, the expression of ligands and receptors in tumor cells may downregulate cell adhesion, thereby favoring the dissemination of metastases, while the expression of the ligands B61 and Lerk2 in the vasculature [31,32] and Htk-L in the bone marrow [44] may influence the targeting of metastatic cells expressing Eph receptors. Because of the existence of so many Eph receptors (and of alternatively spliced forms), the redundancy in ligand–receptor interactions, and the possible importance of the spatial coordinates of their signaling activities, elucidating how the Eph receptors signal will be a particularly challenging task.

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REVIEW

Growth factors acting via endothelial cell-specific receptor tyrosine kinases: VEGFs, Angiopoietins, and ephrins in vascular development

Nicholas W. Gale[1] and George D. Yancopoulos

Regeneron Pharmaceuticals, Inc., Tarrytown, New York 10591-6707 USA

The term 'vasculogenesis' refers to the earliest stages of vascular development, during which vascular endothelial cell precursors undergo differentiation, expansion, and coalescence to form a network of primitive tubules (Risau 1997). This initial lattice, consisting purely of endothelial cells that have formed rather homogenously sized interconnected vessels, has been referred to as the primary capillary plexus. The primary plexus is then remodeled by a process referred to as angiogenesis (Risau 1997), which involves the sprouting, branching, and differential growth of blood vessels to form the more mature appearing vascular patterns seen in the adult organism. This latter phase of vascular development also involves the sprouting and penetration of vessels into previously avascular regions of the embryo, and also the differential recruitment of associated supporting cells, such as smooth muscle cells and pericytes, as well as fibroblasts, to different segments of the vasculature (Folkman and D'Amore 1996; Lindahl et al. 1997). The adult vascular network is comprised of large arteries, internally lined by endothelial cells and well ensheathed by smooth muscle cells, that progressively branch into smaller and smaller vessels, terminating in precapillary arterioles that then give rise to capillaries. Capillaries are comprised almost entirely of endothelial cells that are only occasionally coated by a smooth muscle cell-like pericyte. Capillaries then feed into postcapillary venules that progressively associate into larger and larger venous structures; venous structures are fully enveloped by smooth muscle cells, though not to the same degree as arterial structures.

The development of a functioning vascular network requires a remarkable degree of coordination between different cell types undergoing complex changes, and is exquisitely dependent upon signals exchanged between these cell types. Vascular endothelial growth factor (VEGF-A) provided the first example of a growth factor specific for the vascular endothelium, and VEGF-A has since been shown to be a critical regulator of endothelial cell development. Not surprisingly, the specificity of VEGF-A for the vascular endothelium results from the restricted distribution of VEGF-A receptors to these cells. The need to regulate the multitude of cellular interactions involved during vascular development suggested that VEGF-A might not be alone as an endothelial cell-specific growth factor. Indeed, there has been a recent explosion in the number of growth factors that specifically act on the vascular endothelium. This explosion involves the VEGF family, which now totals at least five members. In addition, an entirely unrelated family of growth factors, known as the Angiopoietins, recently has been identified as acting via endothelial cell-specific receptors known as the Ties. Most recently, particular members of the very large ephrin family have been identified as having unique roles on endothelium, and at least in some cases appear to act via a receptor that is not only largely restricted to the vascular endothelium but to the endothelium lining venous as opposed to arterial vessels.

As we describe in detail below, a variety of studies highlighted by the analyses of knockout mice (as summarized in Table 1) have implicated the VEGFs, the Angiopoietins, and the ephrins as critical players in particular aspects of vascular development.

The VEGF family

A series of recent papers provide excellent reviews of the VEGF family (Carmeliet and Collen 1999; Dvorak et al. 1999; Eriksson and Alitalo 1999; Ferrara 1999; Neufeld et al. 1999), so we offer only the basics herein. There are five characterized VEGF relatives in mammals (VEGF-A through VEGF-D, as well as PlGF), and they display differential interactions with three related receptor tyrosine kinases (VEGFR-1/Flt-1, VEGFR-2/KDR/Flk-1, and VEGFR-3/Flt-4) as summarized in Figure 1A; although not illustrated, ancillary receptor components, such as the neuropilins, also appear to be involved in these receptor complexes. VEGFR-1 and VEGFR-2 are restricted

[1]Corresponding author.
E-MAIL nick.gale@regpha.com; FAX (914) 347-5045.

Table 1. *Knockout animals from the VEGFR/VEGF, Tie2/Ang, and Eph/ephrin families have exhibited a variety of embryonic defects in vascular development*

Gene knockout	Time of death	Stage of vessel development	Causes of lethality
VEGF-A (+/−)	E11.5	vasculogenesis/ (angiogenesis)	reduced red blood cells; defective heart and aorta formation; defective vessel connectivity; defective sprouting
VEGF-A (−/−)[a]	E10.5	vasculogenesis	absent dorsal aorta; defective endothelial cell development
VEGFR-1	E8.5–E9.5	vasculogenesis	failure of endothelial cell formation
VEGFR-2	E8.5–E9.5	vasculogenesis	excess endothelial cells form abnormal vessel structures entering vessel lumens
VEGFR-3	E10.5–E12	vasculogenesis	defective vessel remodeling and organization; irregular large vessels with defective lumens
Ang1	E10.5	angiogenesis	defective vessel remodeling, organization, and sprouting; heart trabeculation defects
Ang2	E12.5–P1	maturity	poor vessel integrity, edema, and hemorrhage
Tie1	E13.5–P1	maturity	poor vessel integrity, edema, and hemorrhage
Tie2	E10.5	angiogenesis	defective vessel remodeling, organization, and sprouting; heart trabeculation defects
ephrin-B2	E10.5	(vascuolgenesis)/ angiogenesis	some defective vessel primordia; defective vessel remodeling, organization, and sprouting; heart trabeculation defects
EphB2/EphB3	E10.5 (~30%)	(vascuolgenesis)/ angiogenesis	some defective vessel primordia; defective vessel remodeling, organization, and sprouting; heart trabeculation defects
EphB4	E10.5	?	?
EphA2	viable	—	—

Knockouts of VEGF and its receptors have yielded defects primarily in the early process of vasculogenesis, and accordingly these mutant embryos tend to die at early stages of development. In contrast, The Tie2 and Angl knockout embryos die at later stages and exhibit defects similar to each other, but with normal vasculogenesis, and perturbed angiogenesis. Deletion of Ang2, the putative natural antagonist of Angl/Tie2 signaling, results in lethality at stages after the vascular system has undergone both vasculogenesis and angiogenesis. EphB2/B3 and *ephrin-B2* knockout animals die primarily due to defects in angiogenesis similar to the *Tie2* and *Angl* knockout embryos, although some defects in vasculogenesis were also reported. EphB4 mice die at similar stages. (VEGF-A) Carmaliet et al. (1996); Ferrara et al. (1996); (VEGFR-1) Fong et al. (1995); (VEGFR-2) Shalaby et al. (1995, 1997); (VEGFR-3) Dumont et al. (1998); (Angl) Davis et al. (1996); Suri et al. (1996); (Ang2) C. Suri, J. McClain, M.V. Simmons, T.N. Sato, and G.D. Yancopoulos (in prep.); (Tiel) Puri et al. (1995); Sato et al. (1995); (Tie2) Dumont et al. (1994); Sato et al. (1995); (EphB2/EphB3) Orioli et al. (1996); Adams et al. (1999); (ephrin-B2) Wang et al. (1998); Adams et al. (1999); (EphB4) M. Aguet (in prep.); (EphA2) Chen et al. (1996).

[a]Because of the heterozygous lethality of heterozygous VEGF mutations, VEGF null (−/−) mice were generated by ES cell aggregation with tetraploid blastocysts resulting in contribution of the $VEGF^{-/-}$ cells to the embryo proper and not extraembryonic tissues. This difference in technology makes direct comparisons with the knockouts difficult. It might be predicted that contributions of VEGF from the extraembryonic ($VEGF^{+/+}$ but tetraploid) tissues may have dampened the severity of the phenotype and allowed survival of the embryos to later stages of development.

largely to vascular endothelium in their expression, accounting for the specificity of action of this growth factor family. Interestingly, although VEGFR-3 is restricted largely to lymphatic endothelium (Kukk et al. 1996), mice that contain a knockout of the gene for VEGFR-3 display early embryonic lethality due to defects in the organization of large vessels prior to the emergence of lymphatics (Dumont et al. 1998).

The roles of VEGF-A and its receptors, VEGFR-1 and VEGFR-2, have been characterized in the most detail. In vitro, VEGF-A seems to have the ability to induce endothelial cell proliferation as well as migratory and sprouting activity, and to help promote endothelial cells to form tubule-like structures; these effects seem to be mediated largely by the VEGFR-2 receptor. Consistent with these in vitro actions, VEGF-A and VEGFR-2 are absolutely critical for the earliest stages of vasculogenesis in vivo, as blood islands, endothelial cells, and major vessel tubes fail to develop in appreciable numbers in embryos lacking either VEGF-A or VEGFR-2 (Shalaby et al. 1995; Carmeliet et al. 1996; Ferrara et al. 1996). Interestingly, even loss of a single *VEGF-A* allele results in embryonic lethality, demonstrating a remarkably strict dose-dependence for VEGF during development (Carmeliet et al. 1996; Ferrara et al. 1996). Because embryos heterozygous for the *VEGF-A* gene disruption are less affected than those homozygous for this mutation, analysis of these heterozygous mutant embryos has permitted insights into the continuing requirement for VEGF-A as development proceeds. This analysis has revealed that VEGF-A is involved not only in the very initial phases of vasculogenesis, but in later stages of vasculogenesis, in sprouting, and other aspects of angiogenesis, as well as in maintaining vessel survival (Carmeliet et al. 1996; Ferrara et al. 1996).

Mice lacking VEGFR-1 revealed a rather late role for

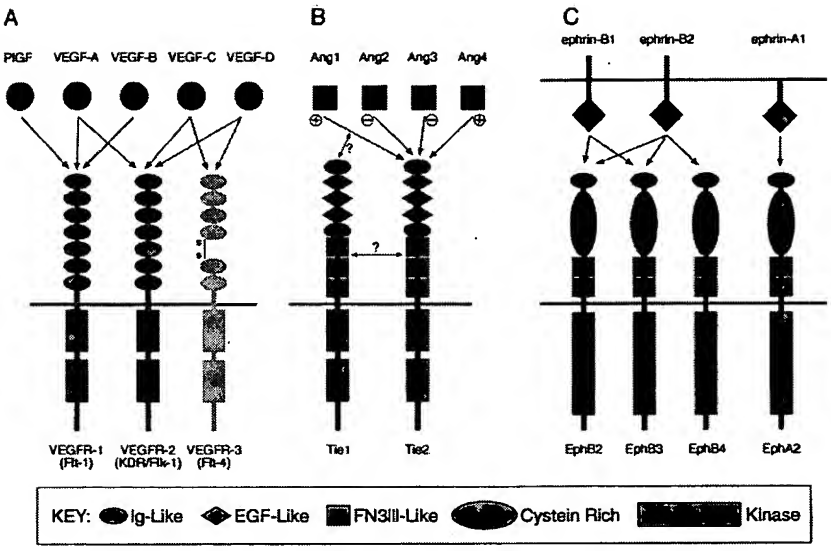

Figure 1. Ligands and RTK families involved in vascular development. (*A*) VEGFs and the VEGF receptors. (*B*) The Angiopoietins (Ang) and Tie receptor family; (*C*) The ephrins and Eph receptors. Arrows indicate documented interactions among ligands with their receptors. Note that receptor and ligand structures are drawn color coded to indicate their domains of expression; purple represents proteins found expressed on both arteries and veins. Red and blue structures are found on arteries and veins, respectively; yellow denotes expression in lymphatic vessels, and green structures represent receptors or ligands expressed in tissues surrounding blood vessels. Structural motifs present within the receptor families are indicated (not to scale) by the key. (FNIII) Fibronectin type III. Receptors of the Tie and VEGFR families exhibit kinases with intervening kinase inserts. The immunoglobulin-like (Ig-like), or globular domain, of the Eph family receptors recently has been crystalized and shown to comprise a jellyroll β sandwich motif (Himanen et al. 1999).

this receptor as compared to VEGFR-2, as vessels do form in these mutant embryos, but are organized abnormally, with seemingly excess levels of endothelial cells being generated and entering into the lumens of the abnormal vascular channels (Fong et al. 1995). Thus, VEGFR-1 may actually be involved in down-regulating VEGF activity to ensure that the right numbers of endothelial cells are generated. Remarkably, mutant mice containing a VEGFR-1 lacking its tyrosine kinase domain allow for normal vascular development (Hiratsuka et al. 1998), indicating that only the binding portion of this receptor may be necessary to support its major actions during vascular development.

The Angiopoietin family

Long after the discovery of VEGF-A, a second family of growth factors specific for the vascular endothelium was identified, with members of this family termed the Angiopoietins (Davis et al. 1996; Suri et al. 1996; Maisonpierre et al. 1997; Valenzuela et al. 1999). Similar to VEGF, the specificity of the Angiopoietins for the vascular endothelium results from the restricted distribution of the Angiopoietin receptors, Tie1 and Tie2, to these cells. Tie1 and Tie2 are receptor tyrosine kinases just as are the receptors for VEGF. The four known Angiopoietins all bind to Tie2, but it is still unclear as to whether they utilize the closely related receptor Tie1 (Fig. 1B). The Angiopoietins provide the first vertebrate example of a growth factor family consisting of both receptor activators as well as receptor blockers, with Angiopoietin-1 (Ang1) and Angiopoietin-2 (Ang2) providing the best characterized examples of an activator and a blocker in this system (Fig. 1B). The existence of both activators and blockers of the Tie2 receptor suggested early on that turning off this receptor might prove to be just as important as turning it on.

The actions of the Angiopoietins appear to be quite different from those of VEGF. Most notably, in vitro analyses on cultured endothelial cells revealed that Ang1 could not elicit standard mitogenic responses as can VEGF-A, nor induce tubule formation, although it could apparently promote endothelial cell sprouting in vitro (Davis et al. 1996; Koblizek et al. 1998). Consistent with these in vitro results, in vivo studies reveal that Ang1 seems to act in complementary and coordinated fashion with VEGF, having a later role in vascular development. Thus, in mouse embryos lacking either Ang1 or Tie2, the early stages of VEGF-dependent vascular development appear to occur rather normally, resulting in the formation of a primitive vasculature (Dumont et al. 1994; Sato et al. 1995; Suri et al. 1996). However, remodeling and stabilization of this primitive vasculature is severely perturbed, leading to embryonic lethality (Sato et al. 1995; Folkman and D'Amore 1996; Suri et al. 1996); defects were particularly obvious in the capillary plexi of the yolk sac and head. These defects are thought to result from disruptions in Ang1-mediated interactions between endothelial cells and surrounding support cells such as smooth muscle cells; support cells produce the Ang1 that paracrinely acts on endothelial cells expressing Tie2 receptors. Ultrastructural examination of vessels in mice lacking Ang1 showed that endothelial cells failed to interact and adhere properly to underlying support cells, and that the endothelial cells appeared rounded in appearance and as if they were detaching from underlying support cells and matrix; in the absence of such optimized cellular interactions, vessels apparently failed to

undergo normal remodeling events and apparently were also at risk for subsequent regression. Another major defect in mice lacking Angl or Tie2 involves heart development. Normal heart ventricles exhibit extensive finger-like projections of the myocardial wall, known as trabeculations, which are lined by coronary endothelal cells (endocardial cells), the specialized endothelium of the heart. In embryos lacking Angl or Tie2, myocardial trabeculations fail to form, and the endocardium appears detached from the underlying myocardium, perhaps reflecting poor attachment. A similar heart defect was also reported in mice lacking neuregulin or its receptor (Gassman et al. 1995; Lee et al. 1995; Meyer and Birchmeier 1995; Kramer et al. 1996), which led to the proposal of a required growth factor loop in trabecular formation involving both an endocardial-derived ligand acting on the myocardium, that is, neuregulin, and a myocardial-derived factor acting on the endocardium, that is, Angl (Suri et al. 1996).

Altogether, the defects in mice lacking Angl or Tie2 suggest that this system is critical for normal remodeling, maturation, and stabilization of the developing vasculature. It has been suggested that the Angiopoietins themselves may not be 'instructive' remodeling signals but are simply 'permissive' in that they allow for proper interactions between endothelial cells and supporting cells, resulting in a system that can then properly respond to other cues (Suri et al. 1996). In the absence of proper interactions between endothelial cells and their support cells, defects arise, whether in particular vascular beds or in a developing vascular structure such as the heart. Consistent with the notion that Angl may continue to play a required stabilizing role for mature vessels, Angl seems to be constitutively expressed in the adult. Interestingly, transgenic overexpression of Angl leads to striking hypervascularization, presumably by promoting vascular remodeling events and perhaps by decreasing normal vascular pruning (Suri et al. 1998).

Shortly after the discovery of Angl, the cloning of Ang2 was described (Maisonpierre et al. 1997). Remarkably, although Ang2 bound to the Tie2 receptor as did Angl, it could not activate it. Instead, Ang2 provided the first example of a naturally occurring antagonist for a vertebrate tyrosine kinase. Consistent with the notion that Ang2 acts as a natural antagonist for the Angl/Tie2 interaction, transgenic overexpression of Ang2 during embryogenesis leads to a lethal phenotype reminiscent of that seen in embryos lacking either Angl or Tie2, with severe disruptions in vascular development (Hannahan 1997; Maisonpierre et al. 1997) .

Examination of Angiopoietin expression patterns in vivo suggested a very interesting role for Ang2 at sites of vascular remodeling in an otherwise stable adult vasculature (Maisonpierre et al. 1997). Whereas Angl is expressed widely in normal adult tissues, consistent with it playing a continuously required stabilization role, Ang2 is highly expressed only at sites of vascular remodeling in the adult, notably in the female reproductive tract (Maisonpierre et al. 1997). Detailed localization of Ang2 in the ovary by in situ hybridization revealed that in regions of active vascular remodeling it was either expressed together with VEGF at sites of vessel sprouting and ingrowth (e.g., developing corpus luteum), or in the absence of VEGF at sites of frank vessel regression (e.g., atretic follicles). These expression patterns led to the proposal of a model in which Ang2 plays a facilitative role at sites of vascular remodeling in the adult by blocking a constitutive stabilizing action of Angl, allowing the vessels to revert to a more plastic and unstable state (Maisonpierre et al. 1997). Furthermore, it was suggested that such destabilization by Ang2 in the presence of high VEGF levels primes the vessels to mount a robust angiogenic response reminiscent to that of early embryonic vessels prior to maturation (Maisonpierre et al. 1997). However, such destabilization by Ang2 in the absence of VEGF is instead proposed to lead to frank vessel regression.

Altogether, existing data suggest that VEGF-A and Angiopoietins not only have quite different roles during vascular development, but also very complementary and coordinated roles.

The ephrins

Among the known families of receptor tyrosine kinases (RTKs) and their ligands, the Eph receptors and their ligands the ephrins stand out in many ways (Fig. 1C). The Eph receptors comprise the largest subfamily of RTKs, including at least 14 distinct members in both man and mouse; Eph receptors and ligands have also been described in invertebrates such as *Drosophila* and *Caenorhabditis elegans* (George et al. 1998; Flanagan and Vanderhaeghen 1998). The Eph receptors are quite divergent in sequence from other RTKs and are closely related to cytoplasmic tyrosine kinases within their kinase domains (Hanks and Quinn 1991). Although all the Eph receptors were initially isolated as orphan receptors, lacking known ligands, this situation has changed dramatically over the past 5 years. At least eight Eph receptor ligands—ephrins—have been described. The ephrins, like their receptor counterparts, are also rather unique among RTK ligands. Most notably, they do not function as typical soluble ligands for their receptors but, rather, must normally be membrane attached to activate their receptors (Davis et al. 1994; Gale and Yancopoulos 1997). Membrane attachment seems to promote clustering or multimerization of the ligands, and it is this clustering that seems to be necessary to activate receptors on adjacent cells (Davis et al. 1994). Consistent with this notion, whereas monomeric soluble ligands seem to act as antagonists, artificial clustering of soluble versions of these ligands can allow them to activate their receptors (Davis et al. 1994; Winslow et al. 1995). All of the known ephrins are tethered naturally to the cells in which they are expressed. It is these two different attachment mechanisms, along with shared homology and binding characteristics, that divide them into two subgroups, the ephrin-A and ephrin-B subgroups (Brambilla et al. 1995; Gale et al. 1996a,b; Eph Nomenclature Committee 1997). The ephrin-B subgroup, comprised of three mem-

bers, ephrin-B1 through ephrin-B3, possesses transmembrane as well as highly conserved cytoplasmic domains, whereas the ephrin-A subgroup, comprised of five members, ephrin-A1 through ephrin-A5, is attached to the outer leaflet of the plasma membrane via a glycosylphoshotidylinositol (GPI) anchor. The ephrin-A subgroup exhibit rather general and promiscuous binding to the EphA receptors (EphA1 through EphA8), and the ephrin-B subgroup exhibits a general binding preference for the receptors of the B subgroup (EphB1 through EphB6). There are a few exceptions to these general binding predelictions. Some receptors are even more promiscuous in that they can bind across subgroups, whereas other receptors display unusually restricted specificity. Notable examples of these two exceptions include EphA4, which binds not only the ephrin-A ligands but several of the ephrin-Bs as well (Gale et al. 1996a,b), and EphB4, which selectively binds ephrin-B2 and not the other ephrin-B ligands (Brambilla et al. 1995; Sakano et al. 1996).

A fascinating series of experiments have suggested that the ephrin-B ligands perhaps should be not only thought of as ligands but as signaling receptors as well. This idea arises because when ephrin-B ligands engage Eph receptors, the ephrin-B ligands themselves become tyrosine phosphorlated and presumably activate signaling within the cells in which they are expressed (Holland et al. 1996; Brukner et al. 1997). Although it is not as intuitively obvious that similar bidirectional signaling events may be occurring with the ephrin-A ligands, owing to their lack of a membrane spanning segment, there is some precedent for other GPI-anchored molecules to transduce signals into the cells to which they are attached (for example, see Brown 1993). Together, these findings clearly blur the distinction between receptor and ligand, and complicate things in a most interesting way, being that one must keep in mind the notion that signaling from Eph receptors and their ligands is capable of being a reciprocal bidirectional event between interacting cells.

Because bidirectional signaling apparently occurs between Eph receptors and ephrin-B ligands, and because this coactivation seemingly depends on clustering of both these membrane-bound partners, it is quite intriguing that molecular mechanisms have recently been described that may allow for regulating such clustering. It has been shown that both Eph receptors and ephrin-B ligands have recognition motifs, at their carboxyl termini, that allow for binding of PDZ domain-containing proteins (Hock et al. 1998; Torres et al. 1998; Buchert et al. 1999; Lin et al. 1999). PDZ domains are modular protein-interaction domains that typically recognize short peptide sequences of four or more amino acids at the very carboxyl terminus of target proteins, and are known to be involved in regulating clustering events among themselves and the protein with which they interact. PDZ interactions can cluster both Eph receptors and ephrin-B ligands and also appear to localize the Eph receptors to synapses in the CNS where they may modulate synaptic functions (Hock et al. 1998; Hsueh and Sheng 1998; Torres et al. 1998; Buchert 1999). PDZ binding has not been described previously for other mammalian RTKs or their ligands, although it appears that other RTKs that are clustered at synapses may also bind PDZ proteins (Torres et al. 1998; Buchert et al. 1999).

In addition to their PDZ interaction motifs, Eph receptors contain another motif that may mediate receptor clustering. The carboxyl terminal region of Eph receptors also includes a sterile α motif (SAM) (Schultz et al. 1997). The crystal structure of Eph family SAMs recently have been described and suggest that these are protein–protein interaction modules that may be involved in modulating receptor oligomerization, as well as interaction with cellular signaling complexes (Stapleton et al. 1999; Thanos et al. 1999).

Eph family function during development

Like the Angiopoietins, ephrins seem unlike most other RTK ligands in that they can not induce potent mitogenic responses from target cells, suggesting that they too are involved in other types of biological processes. Ephrins also seem quite unusual in that they are obligated to act in membrane-bound form, restricting ephrin/Eph interactions to sites of direct cell–cell contact. Consistent with the notion of unusual roles for this family of RTK ligands, to date they have been most solidly implicated in the process of neural cell guidance. For example, Eph receptors and ligands seemingly regulate axon guidance events that establish the retinotopic map in the tectum (for review, see O'Leary et al. 1999). They have also been shown to be involved in the guidance of neural crest cells as they navigate through the trunk and branchial regions of the developing embryo (Krull et al. 1997; Robinson et al. 1997; Smith et al. 1997; Wang and Anderson 1997). The Eph family has also been implicated more generally in patterning of the brain and somites, where they are predicted to have roles in regulating cell mixing and establishing boundaries between distinct cellular compartments (Xu et al. 1995, 1996; Durbin et al. 1998; Gale et al. 1996b). By and large these biological actions of the Eph family can be explained as resulting from repulsive interactions between receptor and ligand-bearing cells, or instead a signal that prevents two adjacent cell types (reciprocally expressing and Eph receptor and a cognate ligand) from intermixing across a boundary (Gale and Yancopoulos 1997). Recently, localization of Eph family members to synapses suggests that they may play an important role not only in guiding neuronal processes to their connections, but in continuing to regulate these connections once they have formed (Hsueh and Sheng 1998; Torres et al. 1998; Buchert et al. 1999).

Several recent studies on ligand and receptor knockouts in mice, in conjunction with some earlier in vitro studies, now suggest that some Eph family members play roles during vascular development that are at least as critical as those served by Eph family members during neural development. It is on these roles that this review will next focus, as we attempt to place these roles in

context with those played by the VEGFs and the Angiopoietins.

Early in vitro findings suggesting Eph involvement in vascular biology

The first known ephrin, ephrin-A1 (at that time termed B61), was first isolated as a protein of unknown function that was induced in endothelial cells following treatment with tumor necrosis factor (Holzman et al. 1990), and subsequent expression studies revealed a rather specific pattern of expression of ephrin-A1 in the developing vasculature (Flenniken et al. 1996; McBride and Ruiz 1998). Additional studies showed that although ephrin-A1 had no discernible mitogenic effects on cultured endothelial cells, it could promote their chemotaxis and induce sprouting in a rat cornea pocket assay (Pandey et al. 1995). It was also shown that ephrin-A1 could promote capillary-like assembly of human umbilical vein endothelial cells (HUVEC) in an in vitro tubule-formation assay (Daniel et al. 1996). Similar studies showed that ephrin-B ligands could also induce tubule formation, but only on different class of endothelial cells derived from human renal microvasculature (HRMEC). Thus, whereas ephrin-A1 induces HUVECs but not HRMECs to form tubules, ephrin-B1 does the same from HRMECs but not HUVECs (Daniel et al. 1996). Subsequent studies established that EphB class receptors are capable of discriminating between the density or the extent of ligand oligomerization, and mediate tubule assembly in response to tetrameric versions of ligand, but not dimeric forms (Stein et al. 1998b). Linking ephrins to VEGF and the Angiopoietins, B-ephrins were shown to induce sprouting in the same in vitro assay previously used to demonstrate VEGF and Angiopoietin actions (Adams et al. 1999). Linking ephrins to integrin function, a very recent study shows that B-eprhins can promote attachment of endothelial cells to extracellular matrix components by activating integrin function (Huynh-Do et al. 1999). Because blocking integrin function has been shown to disrupt many different aspects of angiogenesis, it is intriguing to consider that the Eph family can regulate integrin function on endothelial cells, and also tempting to speculate that integrin reguation can also be important for Eph function in neural and developmental settings.

Ephrin-B2 and EphB4 differentially mark arterial and venous endothelial cells

Although the above findings suggested that ephrins might have a role during vascular development, recent knockout studies of ephrin-B2 and EphB2/EphB3 now firmly establish Eph family members as key players in the process of embryonic vascular development (Wang et al. 1998; Adams et al. 1999).

The initial report of an *ephrin-B2* knock out mouse created a great stir among Eph researchers as well as those working in the field of vascular biology (Wang et al. 1998). In this study the *ephrin-B2* gene was replaced by the *lacZ* gene, thus providing an excellent marker for precisely following the expression of *ephrin-B2*. *lacZ* expression analysis revealed that ephrin-B2 specifically marked arterial endothelial cells at the earliest stages of vascular development (Wang et al. 1998). This finding sparked examination of the expression patterns for EphB receptors, which revealed that EphB4 (which only binds to ephrin-B2, see above) specifically and reciprocally marks only the venous endothelium (Wang et al. 1998). Furthermore, the embryos lacking ephrin-B2 displayed severe defects in the vascular remodeling, subsequent to the initial stages of vasculogenesis, in both arterial and venous domains. It was suggested that the observed vascular defects arose from defects in bidirectional signaling, normally mediated by the reciprocally expressed ephrin-B2 and EphB4, that occurs between arterial and venous vascular beds during embryonic angiogenesis.

The discovery of clear molecular differences in arterial and venulous endothelial cells at such early stages of development, when the vasculature appears in many areas to be a uniform plexus of interconnected tubules in which there is no clear morphological distinction between presumptive arterial and venous domains, was quite unexpected. Though molecular differences in arterial and venule endothelium is clearly noted at later stages, when these cells can be distinguished by differential lectin staining (Thurston et al. 1996), it was conceivable that such differences reflected late-acting physiological influences such as blood flow, pressure, shear forces as well as relative oxygen content. The ephrin-B2/EphB4 expression studies suggest that molecular differences are at least in part programmed genetically in arterial versus venous endothelium, and furthermore that these differences may be critical to normal development of the vasculature.

Vascular defects in mice lacking ephrin-B2, and similarities to defects on mice lacking Ang1 or its receptor Tie2

The ability to distinguish arterial versus venous endothelium at very early stages, which had previously not been possible due to the absence of specific arterial markers, revealed an unexpectedly high degree of interdigitation of arterial and venous vessels in the primary capillary plexus of the early yolk sac of normal embryos (Wang et al. 1998). Interestingly, generation of the primary capillary plexus occurred in the yolk sac of embryos lacking ephrin-B2, and this plexus was still divided in its expression of the newly defined arterial and venous markers. However, interdigitation of arterial and venous vessels in this plexus was absent in mice lacking ephrin-B2, and remodeling of the primary yolk sac plexus into large and small branches also did not occur, consistent with a critical role for ephrin-B2 in these processes. It was also observed that yolk mesenchymal and periendothelial cells appeared to be poorly associated with the endothelium, and exhibited a rather rounded morphology in comparison to wild-type mesenchyme. Here it

appeared as though arterial vessels failed to delaminate from endodermal layers, and in some cases vessels were dilated and appeared to lack the normal complement of supporting cells (Wang et al. 1998). Altogether, the formation of an initial plexus in the yolk sac, coupled with defects in later remodeling that were marked by problems with endothelial cell interactions with support cells, was highly reminiscent of defects observed in mice lacking Ang1 or its receptor Tie2 (Sato et al. 1995; Suri et al. 1996; see above).

Defects elsewhere in the ephrin-B2-deficient embryos were also reminiscent of those in mice lacking Ang1 or its Tie2 receptor. Outside of the yolk sac, these defects were documented most carefully in the primary capillary plexus in the developing head region. In contrast to the yolk sac, the head plexus appeared rather devoid of cells of arterial origin in ephrin-B2 mutants (with the exception of the most proximal branches of the internal carotid artery), and was thus presumably mostly venous in origin. Although some of the venous vessels (e.g., the most proximal branches of the anterior cardinal vein) underwent morphogenesis in ephrin-B2 mutants, these vessels appeared abnormal, with distal branches remaining dilated and unorganized (Wang et al. 1998). These results, like those in the yolk sac, suggest that interactions between arteries and veins (and mediated by ephrin-B2) are important for the normal remodeling of the head vasculature; however the manner in which these interactions occur appears to be fundamentally different than those in the yolk sac. Another aspect of angiogenesis, known as sprouting angiogenesis, is responsible for the ingrowth vessels into previously avascular areas of the embryo, such as the neural tube. In ephrin-B2 knockout animals, no angiogenic sprouts are observed in the neurectoderm. In the case of sprouting angiogenesis into the neurectoderm it was proposed that perhaps vascular spouts of arterial origin (i.e., expressing ephrin-B2) normally interact with EphB receptors, such as EphB2, expressed in the neurectoderm, and that this interaction somehow promotes vessel growth into this tissue (Wang et al. 1998).

Finally, embryos lacking ephrin-B2 displayed defects in heart trabeculation quite analogous to those described in mice lacking Ang1 or neuregulin (Gassman et al. 1995; Lee et al. 1995; Meyer and Birchmeier 1995; Kramer et al. 1996; Wang et al. 1998; Zhao and Lemke 1998). The expression pattern of ephrin-B2 and its receptors in the heart is quite complex and does not conform to a simple arterial/venous distribution pattern, though it remains possible that the distributions are still complementary and that the heart abnormality is due to the same basic defect in cell signaling that accounts for the abnormalities elsewhere in the vasculature. In any case, the realization that ephrin-B2 is also involved in myocardial trabeculation adds another requisite growth factor to this process, but our inability to assign even a sequence to the actions of the three known requisite growth factors (neuregulin, Ang1, and ephrin-B2) emphasizes how little we understand about the process of trabecular formation. Likewise, despite the similarities between many of the vascular remodeling defects resulting from disruption of either ephrin or Angiopoietin signaling, it remains difficult to even order the actions of these two growth factor systems. It seems possible that the Angiopoietin system may act upstream of ephrin-B2, but the reverse is also plausible. However, since Angiopoietins and Ties have not been reported to display asymmetric distributions, it seems unlikely thatAngiopoietins directly establish ephrin-B2/EphB4 expression patterns, or vice versa; nevertheless, it will be fascinating to examine ephrin-B2 expression patterns in the Ang/Tie2 knockouts. It also remains possible that the ephrin and Angiopoietin systems act in largely independent fashions, disruption of either of which leads to a similar phenotype. For example, since it has been proposed that the Angiopoietins themselves may not be instructive but rather permissive, in that they allow for proper interactions between endothelial cells and supporting cells resulting in a system that can then properly respond to other cues, it is possible that ephrin-B2 may be just such an instructive cue that requires prior permissive actions of the Angiopoietins.

The roles of ephrin-B2 and EphB4 in vascular remodeling: developing more complications

Despite the remarkably reciprocal expression of ephrin-B2 and its EphB4 receptors in developing arterial and venous endothelium, it remains completely unclear as to precisely where and how the critical interactions occur between cells expressing these signaling partners (Wang et al. 1998; Yancopoulos et al. 1998). Because both partners are membrane bound, it is presumed that signaling must be occurring at sites of cell–cell contact. Thus, although critical signaling could be occurring within individual tubules precisely at the junction of arterial and venous cells (*cis* interactions, Fig. 2), ephrin-B2/EphB4 interactions could also be occurring as developing tubules pass each other, or as a developing sprout touches an adjacent tubule (*trans*-interactions, Fig. 2).

To complicate matters even further, an independent report of an *ephrin-B2* gene deletion confirmed the overall phenotype initially described by Wang et al. (1998) but raised many additional issues (Adams et al. 1999). The later study raised the question of whether other ligands and receptors in the B subgroup also played crucial roles in vascular development along with ephrin-B2 and EphB4, and whether these B-class receptors and ligands were critical not only for interactions between arterial and venous endothelial cells, but for interactions between endothelial cells and surrounding cells and tissues (Adams et al. 1999). These questions arose because Adams et al. (1999) found that other B-class receptors and ligands displayed expression patterns overlapping those for ephrin-B2 and EphB4, and also found that embryos lacking both EphB2 and EphB3 exhibited defects in vascular development that largely phenocopy those of the ephrin-B2 knockouts (Adams et al. 1999). That is, with a penetrance of ~30%, embryos lacking EphB2 and EphB3 (EphB2/B3) displayed defects similar to those of ephrin-

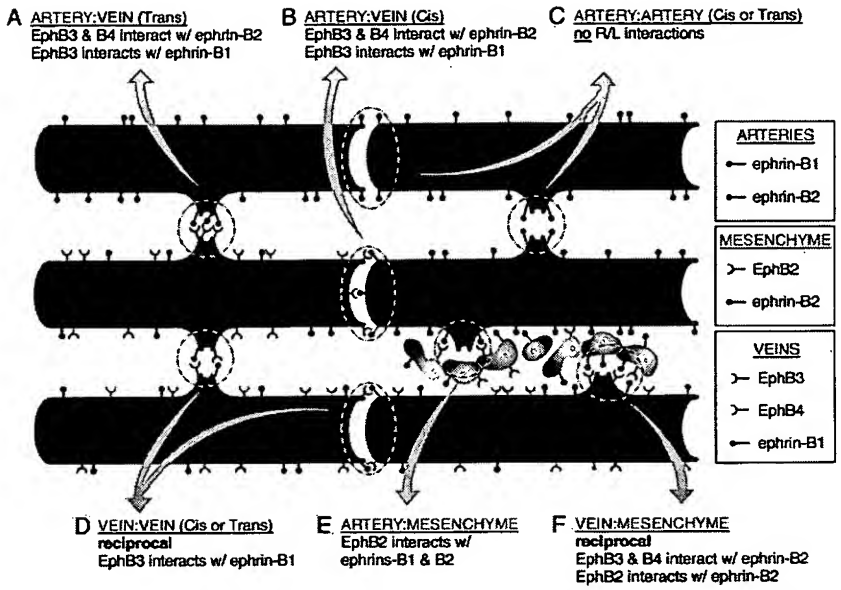

Figure 2. Expected interactions of Eph family members on arteries, veins, and mesenchyme. Arteries express ephrin-B1 and ephrin-B2, whereas veins express EphB3 and EphB4, and ephrin-B1 (see keys). Veins and arteries and the cells within a given vessel can interact with each other in several ways, and these interactions would be expected to bring Eph family members in contact with each other in several ways. Interactions between cells of a given vessel, which we term *cis* interactions (*B–D, center*), will occur among venous cells in veins and arterial cells in arteries. In the special case of capillaries, where vessels switch between arterial and venous domains, arterial and venous cells will also contact one another within a single vessel. Interaction between distinct arterial and venous vessels in regions where they grow closely together or contact each other by sprouting will also bring cells expressing Eph family members into contact with each other in a *trans* configuration (*A,C,D*). Additional important interactions occur when arterial or venous sprouts interact with surrounding mesenchymal cells, which express EphB2 and ephrin-B2 (shown) and most likely other family members as well (*E,F*). It is important to note that the distinction between Eph receptors and their ligands is somewhat arbitrary in that both components are capable of signaling when bound to their counterparts; thus, signals between arteries, veins, and mesenchyme will be bidirectional. In addition, in the case of vein cell interactions with other venous cells, and mesenchymal cells signaling can theoretically be reciprocal, because both interacting cells express receptors as well as ligands.

B2 knockout mice in the primary plexi of the yolk sac and head, and in heart morphogenesis. However, sprouting angiogenesis into the neural tube was normal in EphB2/B3 knockout mice, in contrast to the situation in embryos lacking ephrin-B2.

Whereas Wang et al. (1998) portrayed a relatively simple picture in which arterial endothelium expresses ephrin-B2 and venous endothelium expresses EphB4, and suggested that other B-class receptors and ligands were not involved in this system, Adams et al. (1999) described a much more complex situation. While confirming the remarkable specificity of ephrin-B2 and EphB4, Adams et al. (1999) show that several other B-class receptors and ligands are expressed in and around the developing vasculature. In particular they reported that ephrin-B1 is coexpressed with ephrin-B2 on arterial endothelium (Fig. 2), though its presence there obviously is not sufficient to compensate for the knockout of ephrin-B2. In addition, in a few locations, such as the aortic arches, they reported that the EphB3 receptor is also expressed in arteries. In veins, they claim that EphB3 appears to be broadly expressed and overlaps the expression of EphB4, and that ephrin-B1 is also expressed in veins in addition to its expression in arteries (Fig. 2). Thus venous endothelium seems to generally express both EphB3 and EphB4 receptors as well as an ephrin-B ligand, and arterial endothelium expresses both ephrin-B1 and ephrin-B2 as well as expression of EphB3 in some limited sights [e.g., in aortic arches (Adams et al. 1999)]. To complicate things even further they report expression of Eph family members in tissues surrounding vessels at sites of sprouting angiogenesis, such as in the somites and the neural tube. The remarkably reciprocal expression patterns of ephrin-B2 and EphB4, together with the strict specificity of EphB4 for binding only to ephrin-B2, seem to undeniably support a unique role for this set of partners (Fig. 2). However, the widespread expression patterns of other B-class Eph family members, together with the phenotypes of mice lacking EphB2 and EphB3, indicate that other B-class family members also play critical roles in vascular development. These roles may in some ways overlap with those of ephrin-B2 and EphB4 and, at least for ephrin-B2, may also involve binding to it as well. Furthermore, in addition to *cis* or *trans* interactions between arterial and venous endothelial cells, the expression of B-class Eph family members in surrounding tissues suggests that Eph-mediated interactions between endothelium and surrounding cells might be very critical (Fig. 2). For example, sprouting angiogenesis into somites appears to provide a specific example in which ephrin-B2 expressed not by endothelium, but rather in surrounding mesenchyme, interacts in a critical manner with EphB4 and EphB3 receptors on vessels (Adams et al. 1999). It has been shown that ephrin-B2 is expressed in the posterior half of the somitic sclerotome as well as in the dermomyotome, and its presence there has been demonstrated to be involved in the guidance of both motor axons and neural crest cells which negotiate their way through the sclerotome during development (Krull et al. 1997; Wang and Anderson 1996). Here the ephrin repels these cells or processes from the posterior somite and presumably channels them along their appropriate pathway through the anterior somite (Krull et al. 1997; Wang and Anderson 1997). Such repulsive interactions

may likewise occur in the vasculature, because vessels that develop within/around somites express EphB3 and EphB4 and are observed to grow in close association with, but not within, domains of expression of ephrin-B2 in the dermomyotome in wild type mice. In addition, intersomitic vesses exhibit abnormal projections into the somites in ephrin-B2 knockout mice, where the putative inhibitor is absent (Adams et al. 1999). Likewise embryos devoid of EphB2 and EphB3 exhibit aberrant patterns of sprouting from intersomitic vessels into the neighboring somites (Adams et al. 1999). Because abnormal sprouting into somites is seen in mutants lacking ephrin-B2 or the EphB2/EphB3 receptors, these interactions presumably normally suppress sprouting (Adams et al. 1999). Thus, somitically expressed ephrin-B2 may serve similar repulsive functions in both neural and vascular pathfinding in the region of somites.

Altogether, the findings of Adams et al. (1999) complicate the simple picture initially portrayed by Wang et al. (1998) raising the possibility that many different types of interactions, involving different B-class Eph family members as well as different types of cells, might play critical roles during vascular development (Fig. 2). It is also worth reiterating that all of these interactions are further complicated by the possibility of bidirectional signaling in all cases, making it difficult to understand which cell is sending the signal and which is receiving it.

It is also worth noting that although Adams et al. (1999) confirmed the claim of Wang et al. (1998) that most of the defects in the ephrin-B2 knockout represent abnormalities in angiogenic processes as opposed to earlier vasculogenic processes, Adams et al. also report additional defects that seem to primarily involve vasculogenesis (Table 1). For example, they report defects in major vascular structures formed by vasculogenesis, such as the anterior cardinal vein, the fourth arch of the aorta, and in some cases the dorsal aorta itself; in some severe cases they found that embryos lacking ephrin-B2 had no dorsal aorta or just a single vessel, rather than the normally paired structures (Adams et al. 1999). Furthermore, they reported that many of the embryos lacking EphB2 and EphB3 also had defects in the primordia of major vessels, such as the anterior cardinal vein and the aortic arches.

Despite the remarkably specific expression of EphB4 in venous endothelium, its overlapping expression with other EphB receptors in veins, together with the variable penetrance of the phenotypes displayed by EphB2 and EphB3 knockout mice, raised the question of whether EphB4 was as crucial to vascular development as ephrin-B2. The preliminary answer may soon be provided by the generation of mice lacking EphB4, which are reported to display an early embryonic lethal phenotype (M. Aguet, pers. comm.). One clearly might predict that this phenotype may arise due to vascular defects in angiogenesis (Table 1). A detailed comparison of such defects with those in mice lacking ephrin-B2 and EphB2/EphB3 should prove quite interesting. It will also be interesting to examine mice lacking ephrin-B1 for any vascular phenotypes, which would be predicted due to the widespread expression of this ligand in both arteries and veins.

It is in part surprising that deletion of EphB4 alone results in such a dramatic phenotype, because Eph receptor knockout animals have historically not exhibited overt phenotypes when deleted singly, presumably due to compensation by other family members (e.g., Chen et al. 1996; Henkemeyer et al. 1996; Orioli et al. 1996; Park et al. 1996; N.W. Gale and G.D. Yancopoulos, unpubl.). EphB4 may be a special case due to the high degree of specificity of EphB4 for ephrin-B2. Alternatively it may be due to unique signaling capabilities of EphB4 that cannot be compensated by the overlapping expression of EphB3 in veins.

Conclusions

Three different growth factor systems have been described that act via endothelial cell-specific receptor tyrosine kinases. As might be expected, these three different growth factor systems seem to have very different roles during vascular development. The VEGF system seems to be absolutely required for the earliest stages of vasculogenesis, although it continues to play a critical role during subsequent angiogenesis. VEGF seems able to elicit differentiative, proliferative and chemotactic responses from endothelial cells, as well as to promote the coalescence of endothelial cells into primitive vascular structures. Unlike VEGF, Angl cannot promote mitogenic responses or tubule formation. In fact, Angl seems to come into play after VEGF, by acting to promote vessel branching and remodeling, as well as to promote maturation and stabilization of vessels. Critical to the function of Angl appears to be its ability to optimize interactions of endothelium with surrounding support cells and matrix, thus stabilizing and maintaining vessels, which it appears to constitutively do in the adult. Angl has a natural antagonist, Ang2, which seems to be induced in adult settings of neovascularization, apparently blocking the stabilizing effect of Angl and thus allowing vessels to revert to a more plastic state where they are more responsive to VEGF, but also subject to regression in the absence of VEGF. Existing data suggest that VEGF and Angiopoietins act in a very complementary and coordinated fashion. Like the Angiopoietins, the ephrins also seem to primarily act in later stages of vascular development, though they may also contribute somewhat to formation of vessel primordia. Currently, the most striking aspect of the Eph family in regards to the vasculature is the reciprocal expression of ephrin-B2 and its receptors EphB3 and EphB4 differentially marking arterial and venous endothelium. However, several different ephrins, of both the A and B subgroups, appear to be involved in vascular development and in some cases display overlapping expression with ephrin-B2 and EphB3 and EphB4. Although it is clear that complimentary expression patterns play important roles, sites of overlapping expression of receptors and their ligands, though not well understood at present, will undoubtedly also prove to be mechanistically important.

Though some of the defects seen in mice lacking ephrins and Eph receptors resemble those seen in mice lacking Angl, ordering the actions of ephrins and Angiopoietins, or even understanding if these actions are linked in some way, remains very difficult. Part of the difficulty stems from the lack of mechanistic understanding of Eph family function. It is clear that the Eph family functions in the context of cell–cell contact, and that most of the actions of the Eph family in the CNS can be explained by assuming that they are providing a repulsive signal, or a signal which somehow prevents two adjacent and differentially marked cell types from intermixing; these signals may depend on the ephrin density and degree of clustering, and may involve bidirectional signaling. It will be interesting to see if Eph family function in the vascular system conforms to these emerging views of its function in the nervous system.

A relatively unexplored area of study for these three growth factor families involves their mechanisms of intracellular signaling in endothelial cells, and whether they employ any unique pathways. Of particular interest for the Angiopoietins and ephrins will be the way they may regulate cytoskeletal plasticity, matrix attachment, and intercellular adhesions, as well at the involvement of integrins in these processes.

Precise understanding of how all these various growth factor systems coordinately act during vascular development, and how this process is integrated with growth of the entire embryo, is sure to keep vascular developmental biologists enthralled for years to come.

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